



Application Note

Copy-neutral Loss of Heterozygosity in Cancer

Loss of heterozygosity (LOH) is a common contributor to tumorigenesis, leading to the loss of a wild-type allele and the unmasking of a recessive mutation. Scans of genomic copy number (CN) can reveal LOH due to hemizygous deletions, but LOH can also occur independently of CN change, where one chromosome or chromosomal region has been duplicated and its homologue has been deleted. When LOH occurs without CN change, it is commonly termed copy-neutral LOH.

The Affymetrix® Genome-Wide Human SNP Array 6.0 provides industry-leading CN detection with more than 1.8 million markers, including more than 900,000 SNPs for LOH identification. The ability of Affymetrix SNP arrays to combine CN and LOH detection for the identification of copy-neutral LOH is changing the paradigm for analyzing chromosomal changes in different cancer types. Without LOH detection, traditional bacterial artificial chromosome (BAC) or comparative genomic hybridization (CGH) arrays from other commercial providers only provide half of the picture.

This Application Note describes the frequency and relevance of copy-neutral LOH in a variety of cancer samples, and presents a review of recent publications in which Affymetrix SNP arrays were used to simultaneously study CN and LOH. These publications identify significant and common chromosomal aberrations that cannot be identified when examining CN alone.

Introduction

Cancer samples can exhibit chaotic genomes. By definition, cancer results from an accumulation of genetic alterations that lead a cell population from initiation through promotion and then progression. These genetic alterations include subtle changes, such as small gains and losses and nucleotide substitutions, and more conspicuous alterations, such as changes in chromosomal copy number (CN), translocations and high-level amplifications. These are the cause and effect of impairments in cell cycle regulation leading to errors in replication, recombination and cell division.

Cancer research on the DNA level has historically emphasized CN profiling through cytogenetic techniques and focused molecular analyses, such as microsatellite PCR. Yet significant changes in the genome can occur without changes in chromosomal CN, and the scope of molecular analyses has not offered a feasible approach to genome-wide observations of these events.

Copy-neutral loss of heterozygosity (LOH) represents one example of a genomic abnormality in which no net change in CN occurs, yet the abnormality can contribute to tumorigenesis. Copy-neutral LOH can occur due to duplication of one chromosome segment along with loss of the corresponding homologous region, so that the cell retains two copies derived from one parental source and no copies derived from the other parental source. The acquired homozygosity can contribute to tumorigenesis by activating potential

oncogenes, unmasking mutated tumor suppressor genes or contributing to pathogenicity as a result of altered gene expression due to imprinting.

Copy-neutral LOH events cannot be detected when scanning cancer genomes for CN alone, but they can be detected when viewing CN in parallel with LOH or viewing allele-specific CN. Although the majority of cancer genome screens have focused on CN alone, recent studies combining CN and LOH detection in a single experiment, using Affymetrix Genome-Wide Human SNP Arrays, have revealed a growing list of cancer types that present frequent and recurring copy-neutral LOH.

This Application Note presents a collection of recent articles demonstrating the importance and relevance of copy-neutral LOH in cancer.

Publications

LOH AND FOLLICULAR LYMPHOMA

Follicular lymphoma (FL) is a common type of non-Hodgkin's lymphoma that originates from B-cell lymphocytes and is mostly exclusive to adults. The majority of FL cases present a t(14;18) translocation resulting in the constitutive over-expression of an altered Bcl-2 that blocks apoptosis. While knowledge of genomic changes beyond this translocation has been limited, and prognosis based on genomics is not yet available for this patient cohort, a few CN abnormalities have been associated with the disease, including del6q, del1p32-36, +7, +12 and +X.

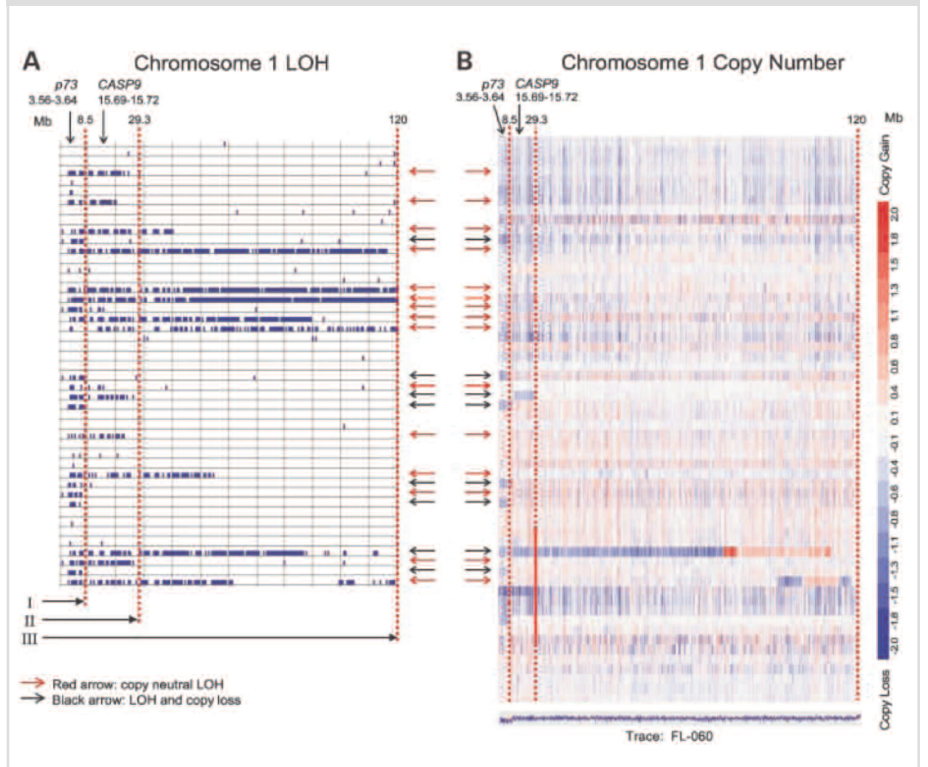
Charles W. Ross¹ and colleagues used Affymetrix SNP arrays² to simultaneously detect CN and genotype in 46 FL samples with paired CD3+ T cell controls. This was the first time that researchers have examined FL samples simultaneously for CN and LOH on a genome-wide level. A number of regions were consistently affected by gains and deletions, as previously seen, but this experiment also identified a number of regions presenting LOH where CN was unaffected.

On chromosome 1p, for example, a region containing p73 and CASP9 exhibited a high frequency of LOH with and without deletion (Figure 1). In total, LOH at chromosome 1 was observed in 50 percent of the samples, becoming the second most frequent genetic lesion ever described in FL. The majority of these LOH events were examples of copy-neutral LOH, with 15 samples exhibiting no CN change and eight samples harboring deletions. By detecting CN alone, only the eight events, 17 percent of all samples, would have been detected, and the significance of this region would have been underestimated. Interestingly, chromosome 1p LOH was the only genetic lesion significantly over-represented in FL grade 2 as compared with grade 1 samples, suggesting the potential differential importance of LOH, including copy-neutral LOH, between these grades.

Chromosome 6p also exhibited a high recurrence of copy-neutral LOH, with 30 percent of FL samples presenting LOH in the affected area. Eighty-six percent of these events were without CN change, indicating a prevalence of 6p copy-neutral LOH. Interestingly, copy-neutral LOH at this site co-occurred with LOH at chromosome 1p.

The authors comment, “Copy-neutral LOH is not detectable using either conventional cytogenetics or array-CGH and, therefore, has not been previously described in FL.” The novelty of these findings, therefore, is rooted in the fact that adequate means to detect these types of changes had simply not been applied to this disease type.

Figure 1: Each row represents one sample; the x-axis represents chromosome position. (A) LOH is indicated by blue for 46 samples, along a stretch of chromosome 1p. **(B)** Copy number is indicated on a scale from red (gain) to blue (loss) for 58 samples. CN for 46 of these samples is aligned horizontally with LOH predictions from the same sample. Red arrows = samples presenting copy-neutral LOH; black arrows = samples presenting LOH with CN loss.



ALLELIC IMBALANCE AND MYELOPROLIFERATIVE DISEASE

Myeloproliferative disease (MPD) is characterized by excess production of cells in the bone marrow. This group of diseases is divided into four main groups: chronic myelogenous leukemia (CML), which contains the Philadelphia chromosome, and three diseases without this translocation. These include Polycythemia vera (PV), Essential thrombocytosis (ET), and Myelofibrosis (MF). A clonal mutation of JAK2 tyrosine kinase (V617F) occurs with high frequency in patients with PV, ET and MF, suggesting a common pathogenesis for the diseases that is negative for the Philadelphia chromosome.

Go Yamamoto³ and coauthors used Affymetrix SNP arrays⁴ to detect CN and LOH across a sampling of MPD cases. The MPD samples were characterized by high

tumor heterogeneity, where CN or LOH events may only affect a minor population within the whole tumor. Because genotyping, by definition, assigns a single genotype across the whole tumor sample, it was not effective at detecting LOH in minor populations of these MPD samples.

Instead, the authors took advantage of allele-specific CN detection, which provides a CN value for separate alleles at a given SNP. When 100 percent of cells have LOH, allele-specific CN values would be “0 and 2” for the two SNP alleles. During retention of heterozygosity, allele-specific CN values would be “1 and 1” for heterozygous SNPs. The utility of allele-specific CN in detecting LOH of mixed samples is that CN values can fall between integers, such that a tumor in which 50 percent of the cells exhibited LOH would display allele-specific CN values of “0.5 and 1.5.”

In Figure 2, Yamamoto, *et al.* demonstrate both the sensitivity of allele-specific CN to mixed populations and the significance of copy-neutral LOH events identified by this method. In Figures 2A and 2B, the same primary acute myeloid leukemia (AML) specimen is studied using paired (Figure 2A) or unpaired (Figure 2B) references. It is clear that the whole of chromosome 17 remains diploid, but copy-neutral LOH of a portion of the tumor sample is detected on chromosome 17p. Because the allele-specific CN values (green and red lines) do not reach 0 and 2, but instead fall somewhere closer to 0.8 and 1.2, it was determined that the copy-neutral LOH event occurred in approximately 20 percent of the tumor population.

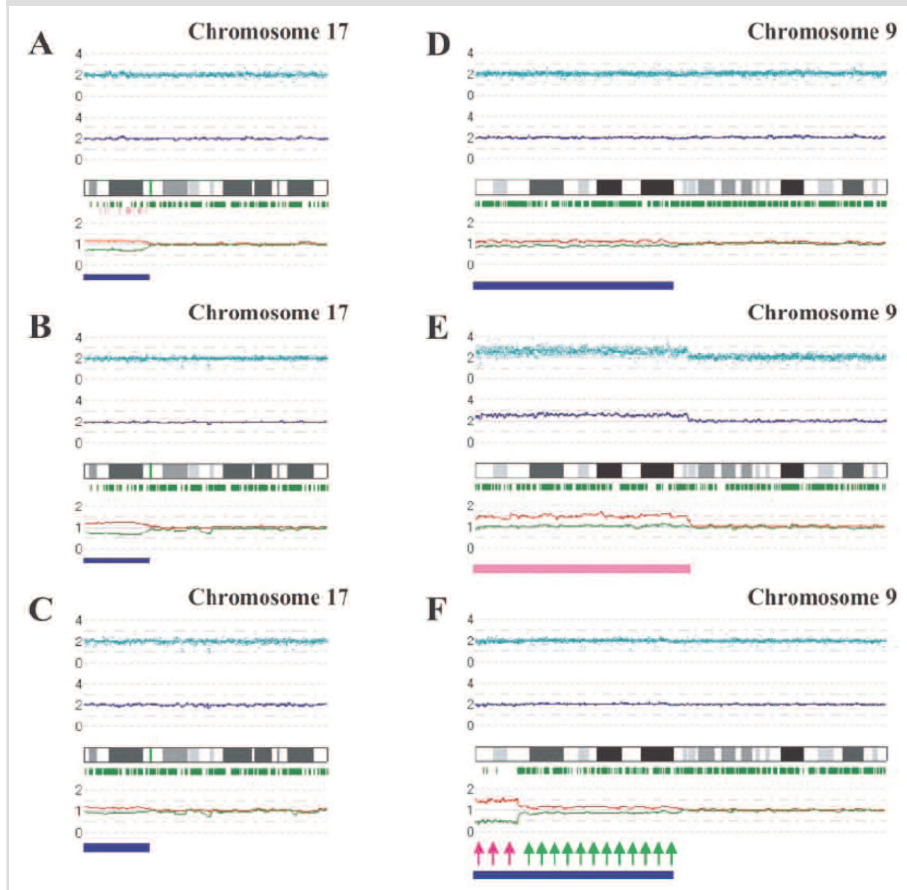
Also of interest: in the pairwise comparison (Figure 2A), less of the population appears to display the duplication than the proportion displaying the deletion (i.e., the red line deviates from CN = 1 less than the green line). This is explained by the fact that a residual tumor component contains the gain but not the loss in the reference sample, which was the bone marrow sample in complete remission (Figure 2C).

The JAK2 gene is located on chromosome 9p, a region with a high frequency of copy-neutral LOH or gain in MPD cases shown to be JAK2 mutation-positive (Figure 2D, E, F). In Figures 2D and 2F, copy-neutral LOH is detected that encompasses the JAK2 mutation, and in Figure 2E, this region displays a duplication. In these examples, only the gain but not the copy-neutral LOH events could have been detected without the aid of allele-specific CN. In addition, this work contributed to the understanding of disease mechanism, as the authors comment that this work demonstrated “how strongly and efficiently a genetic change (point mutation) works to fix the next alteration (mitotic recombination) in the tumor population during clonal evolution in human cancer.”

In total, the authors detected a minor copy-neutral LOH subpopulation in 63 percent of the MPD cases that were JAK2 mutation positive. Interestingly, the preva-

Figure 2: Allelic imbalance in AML samples (A-C) and MPD samples (D-F) were detected.

For each image, the top panel of blue dots represents raw and unsmoothed CN values across the chromosome. The second panel showing a dark blue line is smoothed CN. Below the cytoband, green notches represent heterozygous SNP genotype calls and pink notches represent conflicting genotype calls between paired tumor and normal samples. On the bottom panel, red and green lines represent smoothed allele-specific CN for each of two SNP alleles. The blue bar at the bottom indicates regions of LOH while the pink bar at the bottom indicates a gain. (A) AML sample compared to paired normal displays copy-neutral LOH on chromosome 17p in a portion of the tumor population. (B) The same AML sample displays the same copy-neutral LOH but in an unpaired analysis to anonymous reference samples. (C) Residual tumor component is identified in the bone marrow sample that had been used as the paired reference in part A. (D - F) Allelic imbalance is detected in JAK2 mutation-positive MPD samples. (D) 9p copy-neutral LOH was detected in approximately 20 percent of the population. (E) Allelic imbalance due to a duplication of 9p was detected. (F) Two discrete populations displaying copy-neutral LOH were detected in a single sample, such that the majority of cells exhibiting copy-neutral LOH had a small region affected (pink arrows) whereas copy-neutral LOH extended further down chromosome 9 for a minority of affected cells (green arrows).



lence of copy-neutral LOH differed between MPD diseases, with chromosome 9p copy-neutral LOH present in 100 percent of PV cases and 90 percent of IMF cases, but only 27 percent of ET cases.

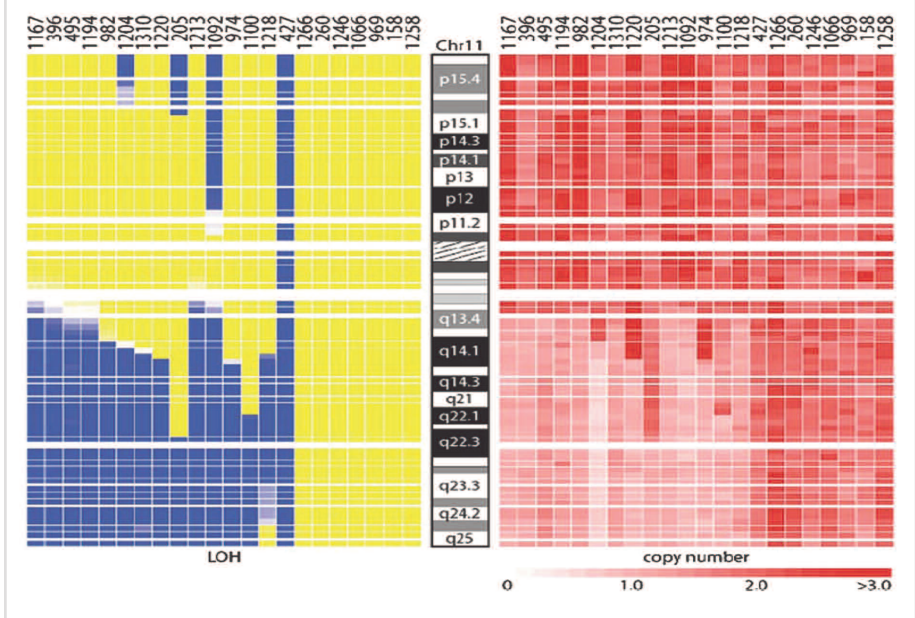
COPY-NEUTRAL LOH IN NEUROBLASTOMAS

Neuroblastoma comprises 6 to 10 percent of all childhood cancers and results in 15 percent

of cancer deaths in children. The cause remains unknown, but it usually begins in the adrenal gland, or may stem from the neck, chest or spinal cord. For most patients the disease has spread by the time of diagnosis.

Rani E. George⁵ and colleagues compared primary neuroblastoma samples with paired blood from 22 children to detect somatic CN and LOH events across the genome⁶. LOH

Figure 3: A total of 22 neuroblastoma tumors were characterized for both LOH (left) and CN (right) on a single SNP array. Blue = LOH; yellow = heterozygosity retained. On chromosome 11p, four samples displayed LOH without change in CN. In contrast, on chromosome 11q, 15 samples displayed LOH, 14 of which also showed an accompanying hemizygous deletion.



was common and found to be recurring in a number of regions, including chromosomes 11q, 3p and 1p. The majority of these LOH regions were associated with a reduction in CN, indicating that homozygosity arose due to a hemizygous deletion. Additionally, one of 15 samples exhibiting LOH on 11q lacked an associated CN change.

some 11p “may result in gain of function of the genes in this region, as for example, IGF2, which is known to induce neuroblastoma cell proliferation. Alternately, the targeted gene within this region on the duplicated allele may also contain inactivating mutations or be suppressed by epigenetic mechanisms, resulting in loss of

“Sequence analysis confirmed that the inactivating NF1 mutation was present on both alleles in all cases of copy-neutral LOH.”

Chromosome 11p also displayed frequent LOH, but in this region neither deletions nor gains were detected. Instead, for the four neuroblastoma samples exhibiting LOH on 11p, all remained diploid across the chromosome arm, indicating that frequent 11p LOH always occurred in the form of copy-neutral LOH for these samples (Figure 3).

The authors state that the recurring copy-neutral LOH regions on chromo-

some 11p “may result in gain of function of the genes in this region, as for example, IGF2, which is known to induce neuroblastoma cell proliferation. Alternately, the targeted gene within this region on the duplicated allele may also contain inactivating mutations or be suppressed by epigenetic mechanisms, resulting in loss of

ADDITIONAL PUBLICATIONS

JUVENILE MYELOMONOCYTIC LEUKEMIA⁷

Juvenile myelomonocytic leukemia (JMML) cells are generally affected by deregulation of the RAS pathway through several possible mechanisms. In approximately 11 percent of cases, RAS hyperactivity is caused by inactivation of the NF1 tumor suppressor gene, which resides on chromosome 17q. In Flotho, *et al.* (*Oncogene*, 2007), an assortment of JMML samples was studied to detect common regions of allelic gains and losses.

In addition to revealing numerous sporadic aberrations that had not been previously identified for this cancer type, these experiments uncovered large regions of copy-neutral LOH on chromosome 17q in 80 percent of patient samples with the NF-1 mutation. Sequence analysis confirmed that the inactivating NF1 mutation was present on both alleles in all of these cases. In contrast, copy-neutral LOH was not detected in any of the samples lacking this mutation. The authors write that “these findings underscore that isodisomy is not a coincidental observation in the leukemic genome of patients with NF-1 who develop JMML.”

CUTANEOUS SQUAMOUS CELL CARCINOMAS⁸

Little is known about the genomic causes of cutaneous squamous cell carcinoma (SCC), the second most commonly diagnosed cancer type in fair-skinned populations. In this study, Purdie, *et al.* (*Genes, Chromosomes & Cancer*, 2007) identified recurring patterns of chromosomal alterations including loss, gain and LOH. The most frequent aberration detected was that of LOH on chromosome 9p (observed in 81 percent of SCC samples).

Although copy-neutral LOH has never before been detected in SCC samples, the SNP arrays revealed copy-neutral LOH at 9p in 23 percent of these samples. In fact, the researchers found that copy-neutral LOH can be common across SCC samples,

with recurring acquired copy-neutral LOH detected on 2q, 7q, 8p, 9p, 9q, 13, 17q and 18q. The underestimation of copy-neutral

Genes involved in early-acting tumor suppression, such as APC, CDKN2A, MLH1 and MSH2, were present in recurring copy-

neutral LOH across a large cohort of 399 pediatric acute lymphoblastic leukemia (ALL) samples. Whole and/or partial chromosome copy-neutral LOH was observed in approximately 25 percent of cases, with chromosome 9 the most commonly affected chromosome.

“Copy-neutral LOH rendered tumors homozygous for pre-existing mutations in genes including CDKN2A and TP53.”

LOH frequency in SCC is a result of the absence of previous analyses matching CN with LOH in these sample types.

COLORECTAL CANCER CELL LINES*

In this study, Melcher, *et al.* studied colorectal cancer cell lines were studied using both spectral karyotyping (SKY) and Affymetrix SNP arrays (*Cytogenetic Genome Research*, 2007). Both methodologies successfully identified complex chromosomal aberrations, but the SNP arrays revealed additional and frequent copy-neutral alterations representing copy-neutral LOH.

neutral LOH regions. In contrast, genes involved later in the adenoma to carcinoma transition were not affected by copy-neutral LOH. These results are consistent with the model, stating that inactivation of tumor suppressor genes through LOH can contribute to the early stages of disease.

MOLECULAR ALLELOKARYOTYPING¹⁰

In a study by Kawamata, *et al.* (*Blood*, 2007), molecular allelokaryotyping—evaluating chromosomal abnormalities are evaluated with specificity to separate SNP alleles across the genotype—was performed

This is the first study to indicate that copy-neutral LOH across chromosome 9 or 9p is common in pediatric ALL. While JAK2 mutations, located on 9p, are common in myeloproliferative disorders (MPD), this mutation is rare in ALL. Also, these cases were negative for the commonly known JAK2 mutations, suggesting that another unidentified region serves as a basis of mechanism for the 9p copy-neutral LOH effect in ALL.

An interesting aside: most whole-chromosomal copy-neutral LOH cases were concentrated in hyperdiploid ALL samples (HD-ALL), whereas copy-neutral LOH that occurred across only part of the chromosomes was generally detected in non-HD-ALL cases, probably as a result of mitotic recombination rather than mis-segregation.

The Affymetrix Solution for Cancer Research

CN and LOH Detection With two experiments in one, get the most value from a single array	Array	Affymetrix® Genome-Wide Human SNP Array 6.0 Highest coverage for combined CN and LOH detection on a single array
	Assay	Affymetrix® Genome-Wide Human SNP Nsp/Sty Assay Kit 5.0/6.0 Low sample requirement with flexibility on source of DNA
	Software	Partek® Genomics Suite™ Single analysis tool for all applications; capable of simple analysis and complex statistics and integration
Integration Identify regions of highest importance and mechanisms of action by comparing data across the -omics	Gene Expression Exon Expression Alternative Splicing	GeneChip® Human Exon 1.0 ST Array Detects all variations in expression, not just those focused on the 3' end
	ChIP-on-chip	GeneChip® Human Tiling 2.0R Array Set Most comprehensive whole-genome array set for studying protein/DNA interactions in chromatin immunoprecipitation (ChIP) experiments
		GeneChip® Human Promoter 1.0R Array Single array to study protein/DNA interactions in more than 25,500 human promoters
	Transcript Mapping	GeneChip® Human Tiling 2.0R Array Set Leave all assumptions aside—identify all known and unknown transcripts expressed in your samples
		GeneChip® Mitochondrial Resequencing Array 2.0 Provides the entire 16 kb mitochondrial genome sequence on a single array
Sequence	GeneChip® CustomSeq® Resequencing Arrays Flexible custom arrays containing up to 300 kb of unique, high-quality, double-stranded sequence for less than a penny per base	

FOLLICULAR LYMPHOMA¹¹

Transformation to a more aggressive lymphoma (t-FL) is common in patients with follicular lymphoma (FL), but the mechanism of this transformation remains to be defined. Fitzgibbon, *et al.* (*Leukemia*, 2007) assessed the contribution of acquired copy-neutral LOH to the transition of FL to t-FL and identified recurring regions of copy-neutral LOH. Sixty-five percent of LOH events occurred without CN change, and these examples of copy-neutral LOH were present in 88 percent of tumor samples, locating to 16 different chromosomes. Sequence analysis indicated that copy-neutral LOH rendered tumors homozygous for pre-existing mutations in genes including CDKN2A and TP53. These results confirmed previous observations by this group that mitotic recombination follows mutation in these samples, resulting in the unmasking of the mutated gene version.

UPD IN COLORECTAL CANCER¹²

In this analysis of CN and LOH across adenocarcinoma samples (Andersen, *et al.*, *Carcinogenesis*, 2007), half of the LOH events detected occurred with deletion, whereas the remaining half represented copy-neutral LOH, indicating copy-neutral LOH. These copy-neutral LOH regions were focused in specific locations, including 8q, 13q and 20q. Transcriptional analysis of adenocarcinoma samples compared to normal mucosa confirmed increased gene expression associated with CN gains, decreased expression in regions of CN loss and no effect on gene expression in chromosome regions displaying copy-neutral LOH. This implies that these copy-neutral LOH regions influence tumorigenesis not through altered gene expression due to imprinting, but through a change in the allelic representations of the genes.

Conclusion

This Application Note represents nine examples of peer-reviewed publications highlighting the prevalence and significance of

copy-neutral LOH. Consistently, it was determined that copy-neutral LOH was common and recurring in a wide range of cancer types. These LOH events sometimes affected known genes and mutations or at other times suggested regions that may contain novel somatic events contributing to cancer development.

In all cases, the approach of combining genome-wide CN with genome-wide LOH detection was necessary to understand the full spectrum of gross chromosomal changes contributing to disease. Affymetrix SNP arrays provide the ability to determine both whole-genome SNP genotype and CN in a single experiment. The SNP Array 6.0 combines more than 900,000 SNPs for LOH identification with an almost equal number of additional non-polymorphic probes empirically selected for dose-dependence CN prediction. The result is a full 1.8 million markers for CN detection, enabling discovery of small CN changes with high confidence and precise fine mapping of CN breakpoints. The combined high coverage of SNPs and CN probes for combined CN and LOH detection in a single array makes the SNP Array 6.0 the ideal solution for cancer genome studies.

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NOTES

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