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Cancer Drug Target Identification by SAGE, LongSAGE, and Digital Karyotyping

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SUMMARY

Activated oncogenes are required for the initiation and maintenance of the cancer cell phenotype, and, therefore, represent attractive therapeutic targets. Specific inhibition of oncogene products was recently approved for cancer treatment. Oncogene activation often results from genomic amplification. The SAGE (serial analysis of gene expression) method has recently been adapted to the analysis of genomic alterations, including amplifications. As a first step in this direction, LongSAGE, which allows one to localize differentially expressed SAGE tags/mRNAs in the human genome, was devised. Subsequently, the LongSAGE protocol was adapted to the quantitative analysis of copy-number changes in genomic DNA. This new method, named Digital Karyotyping, identifies amplifications at an unprecedented resolution. In this chapter the SAGE-based quantification of gene expression and genomic copy-number changes is described as well as how it might be integrated into the identification of oncogenes and cancer drug targets.

Key Words: Oncogene; cancer therapy; Digital Karyotyping; SAGE; serial analysis of gene expression; drug target; tumor biology; tumor suppressor gene; amplification.

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1. ALTERATIONS IN ONCOGENES AND TUMOR SUPPRESSOR GENES CAUSE CANCER

Cancer is a disease primarily caused by the genetic activation of oncogenes and inactivation of tumor suppressor genes (1–4). In addition, epigenetic inactivation of tumor suppressors by CpG methylation of promoter sequences seems to occur at a substantial frequency (5). In the last 20 yr, numerous oncogenes and tumor suppressor genes have been identified. Oncogenes were initially identified via analysis of retroviruses that cause cancer in mice and chicken (1). These viruses were found to have incorporated cellular genes with mitogenic properties into their genomes. The cellular counterparts of these viral oncogenes turned out to be important components of mitogenic signaling pathways (e.g., *SRC*, *MYC*, *HER2/neu*, *FOS*, *MYB*). In human cancer, these genes, also called proto-oncogenes, are activated by genomic amplifications, translocations (with fusion to foreign promoters or protein-coding sequences), or point mutations.

2. ONCOGENES AS ANTICANCER DRUG TARGETS

Cancer therapy directed at specific, frequently occurring molecular alterations in signaling pathways of cancer cells has been validated through the clinical development and regulatory approval in the recent years (6). An example of an approved therapeutic agent is Herceptin/trastuzumab, a humanized antibody directed against the product of the *HER2/neu* oncogene, which is amplified in approx 20% of advanced breast cancer and encodes a receptor tyrosine kinase. Another example is the small-molecule drug Gleevec/imatinib, which inhibits the BCR-ABL tyrosine kinase and the c-kit tyrosine kinase receptor, and is currently used for treatment of chronic myelogenous leukemia and gastro-intestinal cancer.

It is anticipated that a wave of sophisticated “smart drugs” directed at activated or over-expressed oncogene products identified through genomic and proteomic techniques will fundamentally change the treatment of all cancers (6). Tumor suppressor genes obviously represent more difficult targets because their products are lost or inactive in tumors. However, their loss could be exploited via the resulting increased sensitivity to DNA-damaging treatments or recombinant viruses, for example (7–10). For several oncogenes, it was shown that they are not only involved in establishing the tumor, but that they are also necessary for maintaining the growth and proliferation of tumor cells; for example, conditional inactivation of c-MYC in an experimental c-MYC-induced tumor leads to differentiation of tumor cells (11). Subsequent reactivation of the conditional c-MYC allele induced apoptosis, suggesting that even transient therapeutic inhibition of c-MYC could lead to a significant tumor reduction and even complete regression (reviewed in ref. 12). Although a number of oncogenes, which might serve as potential target proteins for further development of targeted therapeutics, are known, there is a shortage of targets for most of the common cancers. In the case of breast cancer, therapy with the anti ErbB2-antibody Herceptin is restricted to approx 20% of the breast cancer patients who have detectable *HER2* amplifications. Therefore, numerous efforts are ongoing to identify frequently occurring amplifications/mutations present in the remaining 80% of breast cancers.

3. TECHNIQUES USED FOR IDENTIFICATION OF ONCOGENIC AMPLIFICATIONS

A number of different techniques has been used in the past to identify oncogenes. As mentioned earlier, the pioneering work took advantage of the fact that murine and avian

retroviruses had incorporated a number of cellular oncogenes. Several of these genes were shown to have undergone activating alterations in human cancer. Another approach was to directly identify and characterize genetic alterations in human cancer by using cytogenetic methods. This led to the discovery of oncogenic fusion proteins resulting from recurrent breakpoint fusions between different chromosomes. One famous example is the *BCR-ABL* fusion, which occurs in CML (chronic myeloid leukemia) as a result of the fusion of the chromosomal regions 9q34 to 22q11 (13). Other oncogenes were identified as components of cytogenetically detectable amplifications of genetic material in the form of double minutes and homogeneously staining regions (HSRs) (14). The increase of gene dosage by DNA amplification is a common genetic mechanism for upregulating gene expression. DNA amplification has been detected in response to exposure to cytotoxic drugs (15–17) or during tumorigenesis (18). Therefore, amplifications can also occur in response to chemotherapeutic cancer treatment and result in resistance (discussed later in this paragraph). The possible genetic mechanisms of gene amplification are discussed in ref. 19. Interestingly, no instance of amplifications as a tissue culture artifact has been reported (19). The region of amplification is often much larger than the oncogene targeted by the respective amplification event. The regions of amplification can range from several hundred Kbp to several Mbp. The size of the amplification unit often correlates with the tumor type. Small amplified regions, which can contain a single gene (*N-myc*), have been observed in 20% of neuroblastoma and correlate with more aggressive variants of neuroblastoma. *N-myc* amplification is currently used as a powerful marker to predict poor outcome in low-stage neuroblastoma (19). Furthermore, amplification of *N-myc* is used to tailor therapeutic approaches. Larger amplification units usually contain several genes. In these cases, the coamplified genes might not convey any advantage for the affected cell. However, in some cases, syntenic coamplified genes might also play a role in tumor development; for example, the 12q13-14 amplification involves the *MDM2*, *CDK4*, and *GLI* genes, all of which have oncogenic activity when expressed alone. This amplification occurs in sarcomas (20) and in neuroblastoma (21). Another famous example of an amplified oncogene is the *ErbB2/HER2/neu* gene, which encodes a receptor tyrosine kinase (22). Approximately 20–25% of primary breast cancer showed amplified *HER2/neu* genes (23). The level of observed amplifications was up to 20-fold. The *HER2/neu* amplification was found to be a significant predictor of overall survival and time to relapse (24). Generally, levels of amplifications in tumors can range from 5-fold to more than 500-fold. In most tumors, values around 50- to 100-fold are seen.

The development of the comparative genomic hybridization (CGH) method, which allows the genomewide analyses of copy-number changes at a resolution between 10 and 20 Mbp, supported the identification of amplified genomic regions harboring oncogenes. However, because of the low resolution of CGH, only a small number of oncogenes were identified via this route. One example is *PIK3CA* (14). In the future, array-CGH (also referred to as matrix-CGH), which promises to have a resolution up to 50 kbp, will presumably aid in the identification of genomic amplifications and the critical proto-oncogenes involved. However, a number of technical problems have to be overcome before this technique becomes widely used (25). Another approach used for the identification of oncogenes is RDA (representational difference analysis) (26). Recently, *KCNK9* was identified by RDA as a gene amplified 3- to 10-fold in 10% of breast tumors (27).

In rare cases, amplified oncogenes, such as *K-RAS*, were found by analysis of mRNA expression, which allowed the detection of overexpressed gene products (28). Serial analysis

of gene expression (SAGE) analysis of differential gene expression in distinct tumor stages has recently led to the identification of an amplified gene, which can be of therapeutic relevance: Saha et al. analyzed a number of different progression stages of colorectal cancers and found a subset of genes that were dramatically upregulated in metastatic lesions (29). Among these was the gene encoding the tyrosine phosphatase PRL3. Because of the central role of signaling via tyrosine phosphorylation in cancer the *PRL3* gene was studied in more detail. Indeed, 3 out of 12 metastatic tumors analyzed showed a more than 100-fold amplification of *PRL3*. However, gene expression analysis is a rather indirect approach for identifying oncogenes, because cancer cells display a large number of deregulated genes when compared to their normal counterparts. Most of these tumor-specific changes in gene expression are secondary to the genetic and epigenetic changes in a few critical oncogenes or tumor suppressor genes.

4. FROM SAGE TO LONGSAGE: INCORPORATING THE GENOMIC LEVEL

Serial analysis of gene expression was devised to allow the analysis of mRNA expression without prior sequence information of the genes subjected to analysis (30; reviewed in ref. 31). A number of genes and pathways relevant for tumor biology were identified using SAGE (7,31–35). The 14-bp tag, including four fixed positions (CATG), used during conventional SAGE is sufficient to distinguish 1,048,576 different mRNAs. Because human or murine mRNA populations contain 30,000–50,000 different mRNAs, the 14-bp SAGE tag allows one to identify and quantify the correct, corresponding cDNA as shown by numerous validations of differential SAGE-tag expression using independent methods as Northern blotting and quantitative real-time polymerase chain reaction (PCR) (e.g., ref. 36). Nonetheless, some ambiguities occurred when using SAGE because in some cases single SAGE tags match several different mRNAs. Furthermore, the 14-bp tag is not sufficient to map the SAGE tag to the genome and thereby determine position and exons of previously unknown cDNAs (37). cDNAs representing genes expressed at low levels, which might have important regulatory functions, are often not represented in the expressed sequence tag (EST) databases and would be missed in a SAGE screen. Therefore, several techniques using the SAGE tag as a primer in an anchored PCR reaction to identify the cDNA in a gene-by-gene manner were proposed (34,38). It was estimated that approx 15,000 exons have not been confirmed through EST sequencing projects to date (37). Furthermore, calculations showed that a SAGE tag of 21-bp length would provide sufficient information to allow the direct mapping of the SAGE tag to the genome with a certainty of 99.83% (37). Accordingly, the SAGE protocol was modified by changing the type IIS restriction enzyme, which is used to release the LongSAGE tag from the 3' ends of cDNAs, from *BsfmI* to *MmeI*. This allowed one to retrieve SAGE tags of 21-bp size (37). In a pilot experiment, a LongSAGE library of 27,737 tags was generated from a colorectal cancer cell line (37). This library represented 3336 genes annotated in the Human Genome Project. However, an additional 1503 tags matched to exons, which had not been previously annotated, with 583 tags matching to internal exons and 920 to novel genes. In order to validate these results, the expression of 129 candidate genes was determined by reverse transcription (RT)–PCR. Thereby, the expression of 123 predicted genes was confirmed (37). These results show that LongSAGE is a useful tool for the identification of novel genes overexpressed in cancer, which could include tumor markers or drug targets. An overview of the Long-SAGE approach is depicted in Fig. 1.

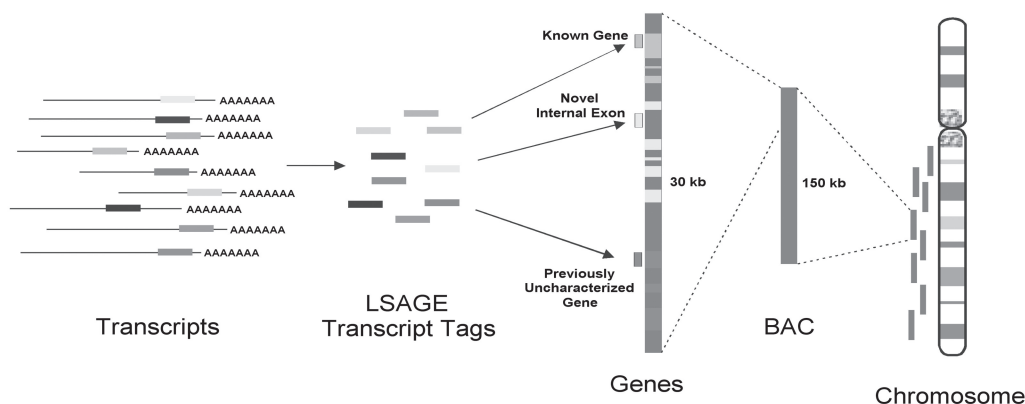


Fig. 1. Schematic of the LongSAGE method. Comparison of tag localizations to previously annotated genes can provide expression evidence for predicted genes and identify novel internal exons and previously uncharacterized genes. *See text for details.* (From ref. 37 with permission from Dr. Victor Velculescu, Nature publishing group: www.nature.com.)

5. DIGITAL KARYOTYPING: CHARTING CANCER CELL GENOMES

As mentioned earlier, the tumor-specific changes in the levels of protein and mRNA expression might be secondary to genetic alterations. Furthermore, gene expression is difficult to measure and might be very specific for each tumor or even the area of the tumor that is analyzed. In contrast, genetic alterations (genomic copy-numbers changes) are stable throughout the tumor cell population. Furthermore, it has been shown that they have a causal role in the development of cancer, because they contain oncogenes (amplifications) and tumor suppressor genes (deletions). Therefore, they provide valuable information about the pathogenetic mechanisms underlying the development of the respective tumor. However, the techniques for detection of these copy-number changes available today are still limited in their resolution. Therefore, Wang et al. recently developed Digital Karyotyping, which is based on the LongSAGE methodology (39) (*see* Fig. 2 for an overview of the Digital Karyotyping procedure [Color Plate 1, following p. 78]). As with SAGE, it is not necessary to clone probes for the sequences that are being analyzed. However, it is necessary that the genome of interest be completely sequenced and aligned. For Digital Karyotyping, the genomic DNA (approx 1 μ g) is cleaved by the so-called mapping enzyme, a restriction enzyme with a 6-bp recognition sequence (e.g., *Sac*I), which results in fragments with an average size of 4096 bp. After ligation of biotinylated linkers to the DNA molecules, a digest with a 4-bp recognizing enzyme (*Nla*III, recognizes CATG) is performed. The DNA fragments containing the biotinylated linker are purified by magnetic streptavidin beads. An oligonucleotide harboring a type IIS restriction endonuclease (*Mme*I) recognition site is ligated to the free *Nla*III site. The LongSAGE tags are then released by cleavage with *Mme*I, which cleaves 21 bp from its recognition site. The released 21-bp tags are self-ligated, PCR-amplified, concatenated, cloned, and automatically sequenced. Sequencing of at least 160,000 tags is necessary to obtain interpretable karyotypes. Because approx 30 LongSAGE tags can be determined per reaction, sequencing of approx 5500 plasmids is required. The templates for these sequencing reactions can either be DNA plasmids

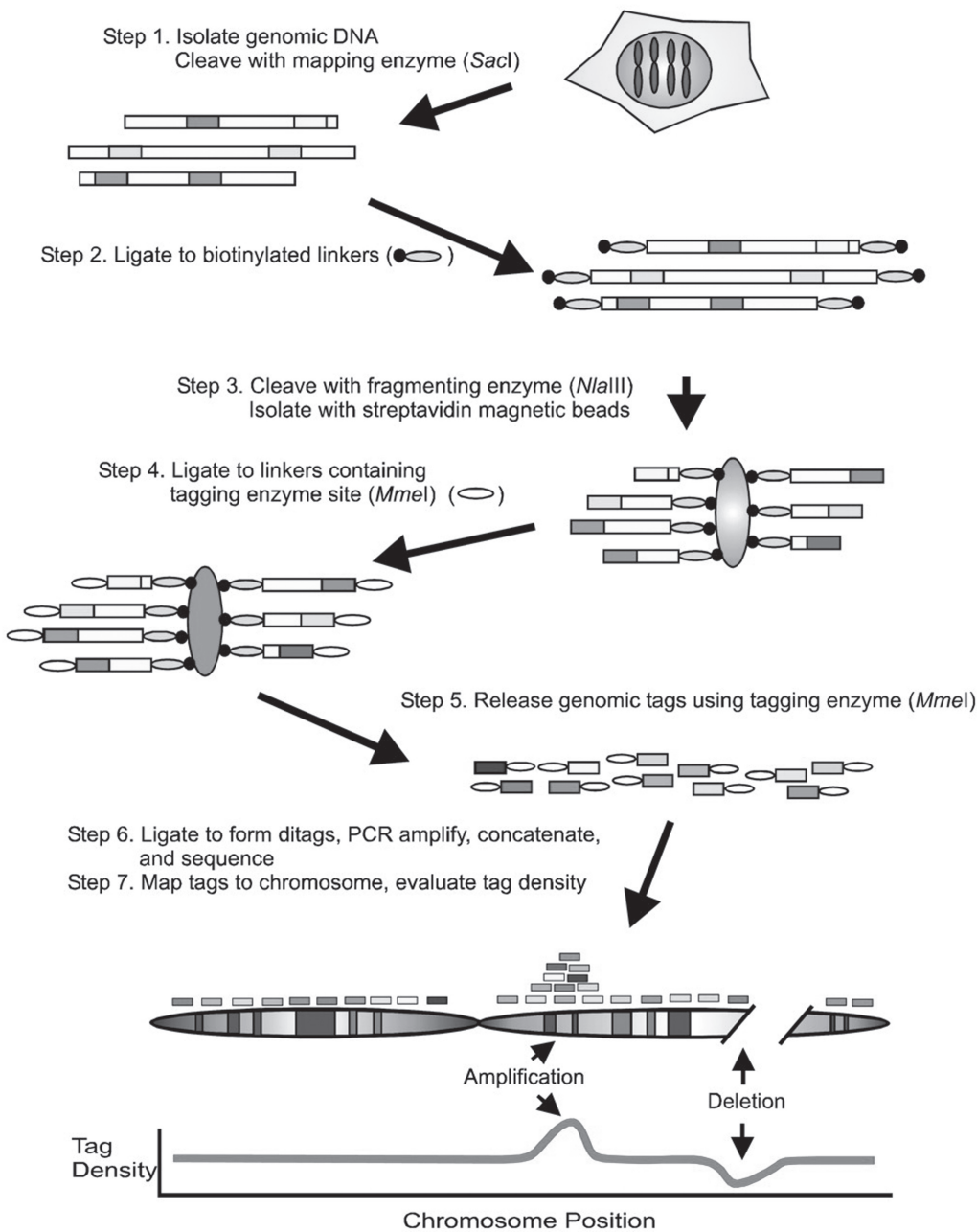


Fig. 2. Summary of the Digital Karyotyping procedure. Small, shaded squares represent genomic tags. Small ovals represent linkers. Large ovals represent streptavidin-coated magnetic beads. See text for details. (See Color Plate 1 following p. 78; from ref. 39 with permission from PNAS.)

or PCR products. By capillary sequencing, this task can be completed in 1–5 d, depending on the availability of a 96- or 384-format sequencer. The resulting sequence data are imported into a so-called SAGE library with the help of the SAGE2000 software and subse-

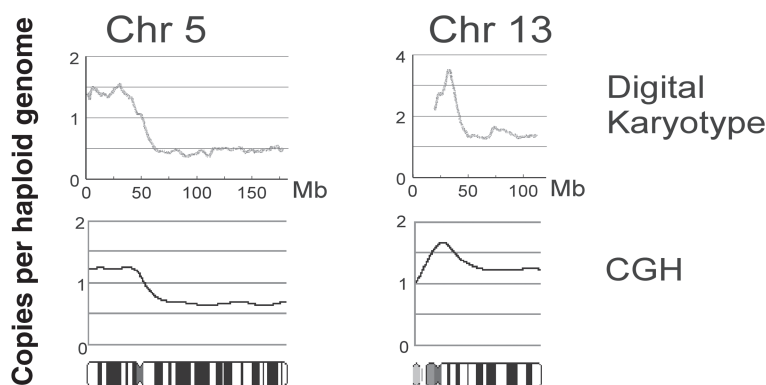


Fig. 3. Digital Karyotyping compared to CGH analysis. Genomic DNA derived from the colorectal cancer cell line DiFi was analyzed either by Digital Karyotyping (upper panel) or conventional CGH (lower panel). Ideograms of the normal human chromosomes 5 and 13 are depicted below the graphs. Values on the y-axis indicate genome copies per haploid genome. Values along the x-axis represent positions along the chromosome (in megabasepairs). Digital Karyotype values represent exponentially smoothed ratios of DiFi tag densities, using a sliding window of 1000 virtual tags normalized to a diploid genome. An example of a smaller window size is shown in Fig. 4. For details, *see* the text. (From ref. 39 with permission from *PNAS*.)

quently matched with a virtual Digital Karyotyping library representing the tags occurring only once in the analyzed genome. In this way, any repetitive sequences are excluded from the analysis. This filtering results in a 40–50% decrease of sequenced tags that are used for the determination of copy-number changes (e.g., 171,795 collected genomic tags resulted in 107,515 filtered tags). The library of filtered tags is then analyzed with Digital Karyotyping software, which generates maps showing the tag densities across the genome. Examples of Digital Karyotyping results compared to conventional CGH analyses of chromosome 5 and 13 of a colorectal cancer cell line are shown at low resolution in Fig. 3. The Digital Karyotyping software allows to display the data at different resolutions and includes a bitmap viewer for maximal resolution and direct access to the raw data at any given chromosomal position. Figure 4 shows the resolution obtained using the bitmap viewer of the amplification on chromosome 7 shown in Fig. 3. The depicted amplification of the *EGFR* (*EGF-receptor*) protooncogene on chromosome 7 has been missed by the CGH analysis, which only indicates a slight increase in the DNA content (data not shown). The size of the amplicon was approx 500 kbp, which explains why it was not detected by CGH. The degree of amplification (approx 120-fold) was confirmed by quantitative PCR (39). Furthermore, the software allows one to set cutoffs for the levels of amplifications and deletions, which should be regarded as relevant. Thereby, the most significant alterations can be rapidly identified. The software described here can be downloaded from the following websites: www.sagenet.org and <http://www.digitalkaryotyping.org/>. The copy-number values obtained by Digital Karyotyping were confirmed by quantitative real-time PCR (qPCR) for a number of amplifications and deletions, suggesting that the quantification achieved by Digital Karyotyping is very accurate (39). Wang et al. estimated the positive predictive value of Digital Karyotyping data at a library size of 160,000 sequenced LongSAGE tags. Accordingly, a 10-fold amplification of only 100 kbp size is reliably

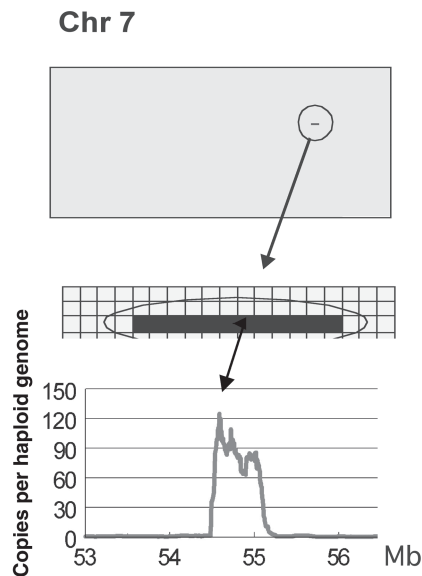


Fig. 4. High-resolution tag density maps of an *EGFR* gene amplification on chromosome 7. A bitmap viewer with the region containing the alteration encircled is depicted. The bitmap viewer is comprised of 39,000 pixels representing tag density values of each virtual tag on chromosome 7, determined from sliding windows of 50 virtual tags. Gray pixels correspond to copy numbers <110, and black pixels represent copy numbers >110. An enlarged view of the region of alteration is shown. (**Bottom**) A graphic representing the amplified region with values on the y-axis indicating genome copies per haploid genome and values on the x-axis representing positions along the chromosome (in Mbp). (From ref. 39 with permission from *PNAS*.)

detected by Digital Karyotyping (39). In case of deletions, the resolution is significantly smaller. The minimal size of reliably detected homozygous deletions is in the range of 500 kbp. Therefore, Digital Karyotyping seems especially useful for the detection of small amplifications. As starting material, 1 μ g genomic DNA is required for an analysis by Digital Karyotyping. Furthermore, several oligonucleotides, modifying and restriction enzymes, PCR and sequencing reagents, and the SAGE and Digital Karyotyping software have to be obtained. The first attempts to perform Digital Karyotyping should be accompanied by a confirmatory CGH analysis of the same tumor DNA, as the CGH results should be very similar to a low-resolution representation of Digital Karyotyping results. Similar to Wang et al. (39), we were able to confirm Digital Karyotyping results by conventional CGH (Hermeking et al., unpublished results).

6. VALIDATION OF DIGITAL KARYOTYPING RESULTS

It is necessary to confirm the alterations detected by Digital Karyotyping using independent methods. In the case of amplifications qPCR or FISH (fluorescence *in situ* hybridization) should be employed to confirm the findings. For qPCR, several primer pairs should be used to confirm each alteration and determine the size of the alteration. Fine mapping the amplification is necessary to restrict the number of potential candidate genes. Generally, even small amplifications will harbor several candidate genes. The genes with

functions similar to known oncogenes (e.g., kinases, transcription factors) represent the most promising candidates for playing a causal role in the detected amplification event. Primer pairs neighboring these genes should be used to screen genomic DNAs isolated from tumors of a larger number of patients (>50) in order to determine the frequency of the gene-amplification event in a particular type of cancer. This analysis will also define the smallest, amplified consensus region. Other types of tumor can be included in the analysis. Several of the classic oncogenes show moderate frequencies of amplification in a given tumor type; for example, *c-myc* and *HER2/neu* are amplified in approx 20% of all breast cancer cases analyzed (19). For certain genes, an alternative to amplification can be activation by point mutation. Therefore, genes found to be amplified by Digital Karyotyping should be analyzed for point mutations in tumors showing no amplification. Interphase FISH using a gene-specific probe can be used to determine the frequency of amplifications in paraffin-embedded tumor sections (40), which allows one to extend the observations made on a few tumors to larger cohorts of archival patient material.

In order to determine which gene is critically involved in the amplification, the encoded gene product should be inactivated by experimental means. For rapid, although partial, inactivation RNA interference (RNAi)-based techniques can be used (41). Recently, a facile approach for the inactivation of specific genes by homologous recombination in human cell lines has been introduced, which can be used in cases where the complete inactivation of the candidate gene is necessary (42). Alternatively, validation by (conditional) knockout approaches in mice can be useful. In Fig. 5, the possible steps and outcomes of a SAGE-based analysis of gene expression and genomic changes in tumors are summarized.

7. FURTHER APPLICATIONS OF DIGITAL KARYOTYPING IN CANCER RESEARCH

Digital Karyotyping has been recently used to show that the gene encoding thymidylate kinase (*TYMS*) is specifically amplified in tumors which underwent treatment with 5-fluorouracil (5-FU) (40). This example shows that Digital Karyotyping can also be useful in identifying genes that modulate the outcome of current cancer therapeutic approaches: Patients with a *TYMS* amplification are largely resistant to treatment with 5-FU and have a considerably worse survival compared to similar patients without *TYMS* amplification (40). Importantly, patients with *TYMS* amplifications, which is relatively easy to routinely detect in paraffin-embedded tumor samples, could be spared from treatment with 5-FU and the associated toxicity in the future. Instead, new drugs specifically targeting the elevated levels/activity of the *TYMS* protein should be developed.

Another application of Digital Karyotyping might be the detection of DNA, which is not normally present in the genome analyzed (e.g., viral DNA) (39). Wang et al. showed that Epstein–Barr virus sequences could be readily detected by Digital Karyotyping of lymphoblastoid cell lines. As more viral genome sequences become available, it might be interesting to determine whether neoplastic cells contain any potentially pathogenic viral DNAs.

8. ALTERNATIVES TO DIGITAL KARYOTYPING

A recent improvement in the resolution of CGH was achieved by incorporating microarrays in the CGH analysis. Instead of hybridizing the tumor DNA to metaphase spreads of normal cells, the DNA is hybridized to BAC (bacterial artificial chromosome)

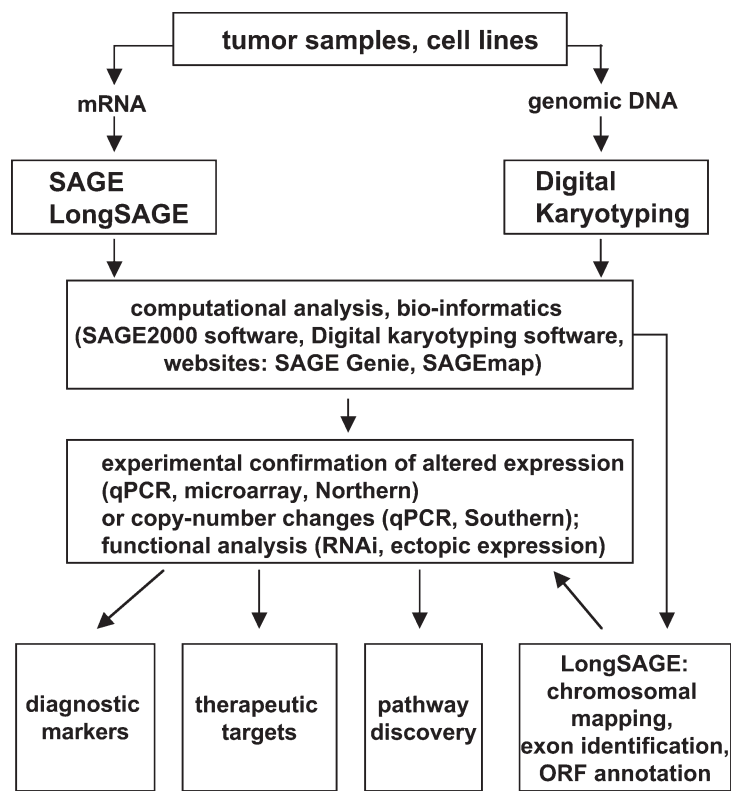


Fig. 5. SAGE analysis of cancer-specific alterations in gene expression and copy-number changes. Flowchart showing the possible steps and outcomes of a SAGE-based analysis of cancer cell genomes and transcriptomes. Website addresses are as follows: SAGEmap: <http://www.ncbi.nlm.nih.gov/SAGE>; SAGE Genie: <http://cgap.nci.nih.gov/SAGE>.

clones representing the human genome spotted on arrays. Dependent on the size and coverage of the BACs used, this technique allows a resolution of 50 kbp. However, currently the use of array-CGH is restricted to a small number of specialized laboratories. Furthermore, there might be significant difficulties because of the problems encountered with hybridizations of complex DNA mixtures.

In the future, a parallel analysis of tumor DNAs by Digital Karyotyping and array-CGH might generate a comprehensive picture of the analyzed cancer cell genome by complementation of the specific advantages of both approaches.

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