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Current Status and Future Clinical Applications of Array-based Comparative Genomic Hybridization

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Introduction

Constitutional chromosomal alterations are commonly detected features in various human diseases such as tumor, congenital anomalies, psychiatric disorders, and metabolic disorders. Neoplastic transformations, for example, are initiated by the aberrations of genes regulating cell proliferation, apoptosis, genome stability, angiogenesis, invasion and metastasis (Hanahan and Weinberg, 2000). Through the population genetics studies, some tumor suppresor genes and oncogenes have been verified (Wakabayashi *et al.*, 2003; Huges *et al.*, 2001; Wilentz *et al.*, 2001; Herranz *et al.*, 1999). And the causative chromosomal alterations for some congenital genetic disorders have been identified by conventional cytogenetic tools. However, there will be even more unknown tumor-related genes, supposedly up to several hundreds, yet to be found (Balmain, 2002), and still a lot of idiopathic psychiatric/ metabolic disorders of unknown origin. In this aspect, precise detection of the breakpoint of chromosomal dosage change, together with the functional and clinical studies, is essential to understand the causes of these disorders and to prevent them. Microarray technology makes it possible to do high-throughput and high-resolution analysis. Combination of conventional comparative genomic hybridization (CGH) and microarray technology promises us genome-wide high-resolution DNA copy number analysis. We review here the recent progress of the array based CGH (A-CGH) technology and its clinical applications.

Historical background of A-CGH

In 1992, Kallioniemi and colleagues first reported CGH to investigate the type and location of DNA copy number changes across whole genome (Kallioniemi *et al.*, 1992). This technique is based on the principle that test DNA and reference DNA, labeled with different fluorescent dyes, are competitively hybridized to normal metaphase chromosomes. The ratio of the two fluorescence intensities detected is indicative of the relative DNA copy number differences in test versus reference DNA. If both genomic DNA have the same allelic copy number, the ratio will be 1. If there is a single copy deletion in one allele, then the ratio will be 0.5 and in the case of copy number gains, the ratio will be 3:2 (single copy gain) or 4:2 (2 copy gain) or more. When the chromosome-wide fluorescence ratio data is combined with chromosome banding data, we can locate the global copy number changes. This concept of CGH introduced a new paradigm of chromosome analysis. Conventional karyotyping needs metaphase chromosome spread from the test tissue. Tissue culture is time consuming and most of the disease tissues are not available for metaphase preparation in practice. Even though we could get the metaphase spread, it is very difficult to interpret and locate the regional gain or loss by karyotyping. CGH technology renders us to overcome the difficulties of karyotyping and improve quality of analysis. For example, commercialized normal chromosome slide could be used instead of a metaphase chromosome from the test tissue. With this new concept, we could also circumvent several limitations of loss of heterozygosity (LOH) analysis, another common approach to analyze the allelic dosage changes. Firstly, CGH enables genome-wide investigation by single hybridization. By LOH analysis, theoretically, several thousands of microsatellite marker PCR and electrophoreses should be performed for the genome-wide analysis with similar resolution. To achieve high-resolution genome-wide screening, LOH analysis is expensive and time- consuming. Secondly, CGH can distinguish between loss and gain of genetic material contrary to LOH analysis. Using CGH technology, a lot of cancer related chromosomal alterations have been identified from various tumors (Mathew *et al.*, 2003; Balsara and Testa, 2002; Buerger *et al.*, 1999; Nessling *et al.*, 1998; Wolf *et al.*, 1999; Ried *et al.*, 1995). However CGH also has its own limitations. The resolution of CGH is not high enough (10-20 Mb) to localize regional chromosome imbalances, which are commonly detected in tumors (Knuutila *et al.*, 1999; Ried

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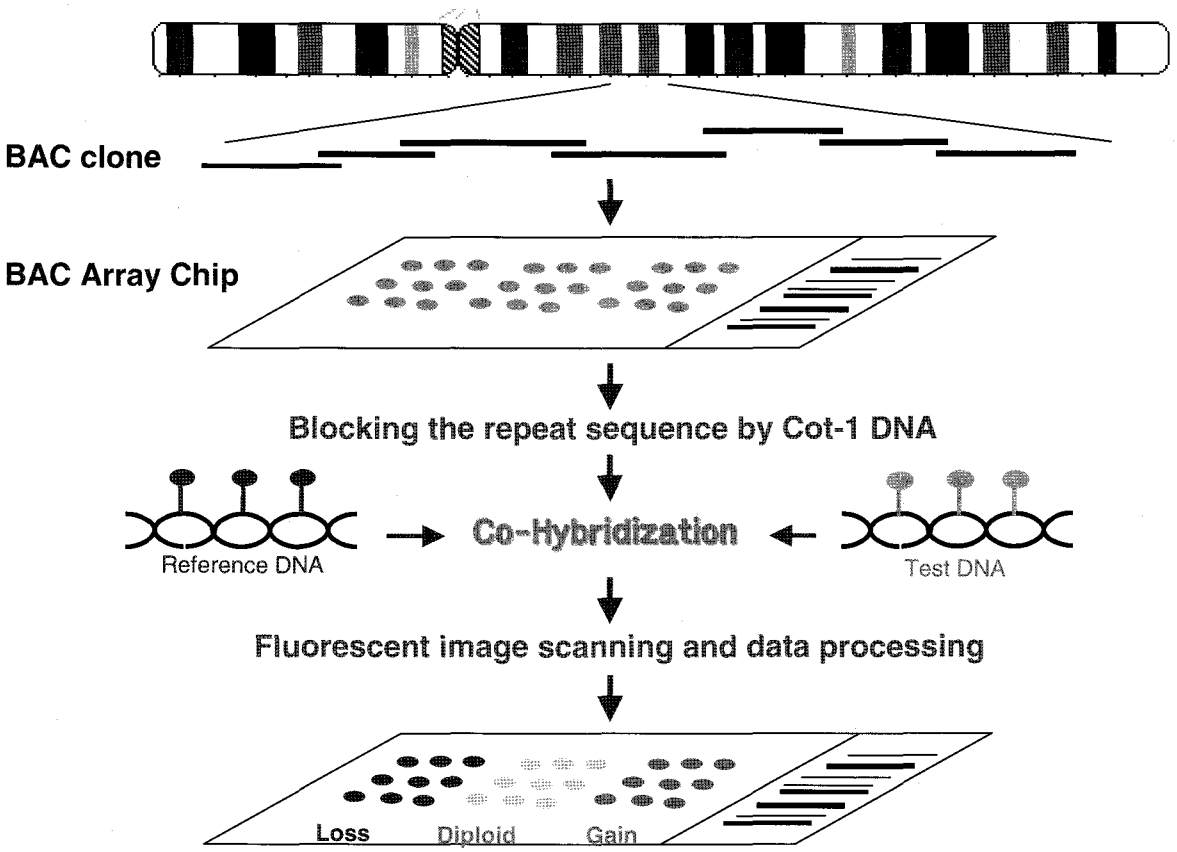

Fig. 1. General strategy of array based CGH. Array CGH chip is constructed by using BAC, PAC, or cosmid resource. Test and reference genomic DNA are labeled with different fluorescent dyes and co-hybridized onto the array slide after blocking the repeat sequences using cot-1 DNA. Fluorescent signals are scanned and processed by using bioinformatics tools. With the dye intensity ratios, DNA copy number alteration can be interpreted and the alteration boundary is measured.

et al., 1999). For scoring the low resolution chromosome banding, researchers need to be highly experienced. Because of these limitations, results from some less experienced laboratories were not trustworthy nor valid. But still CGH is an attractive method to investigate genetic imbalances for tumorigenesis up to now.

Scientists have been attempting to surmount low resolution of CGH and accentuate its advantages by combining CGH and microarray (Pollack *et al.*, 1999; Albertson and Pinkel, 2003). 100-200 kb sized bacterial artificial chromosomes (BAC) clones are used to build tile-path covering all the autosomal and sex chromosomes, from which array chips are produced for CGH analysis (Fig. 1). The use of insert genomic clones such as BACs or PACs for A-CGH provides sufficiently intense signals so that accurate measurements can be obtained for copy number change and direct chromosomal mapping is possible. Recent development of the bioinformatics tools for A-CGH analysis allows more objective and accurate localization of chromosome alterations (Jong *et al.*, 2004; Myers *et al.*, 2004; Wang *et al.*, 2004). Furthermore, since the array format lends itself to automation, it is possible to minimize person-to-person variation. With completion of Human Genome Project draft in 2001 and Mouse Genome Project draft in 2002 making map of BAC clones covering whole genome more accurate and refined, cancer researches using A-CGH are being more facilitated (Pinkel *et al.*, 1998; Hodgson *et al.*, 2001; Snijders *et al.*, 2001; Cai *et al.*, 2002; O'Hagan *et al.*, 2002; Albertson, 2003). Even though A-CGH greatly improved in array production and analysis, it cannot be omnipotent. It is important to remember that A-CGH does not provide information on reciprocal translocation or polyploidy.

Technical consideration of A-CGH

First step to construction of genomic arrays is to prepare the set of BAC clones covering whole human genome. There are several different types of artificial chromosomes

carrying human genome; Bacterial artificial chromosome (BAC), Yeast artificial chromosome (YAC), P1 bacteriophage artificial chromosome (PAC) and cosmid. BAC clone is the most popular resource for A-CGH fabrication. The size of insert is different from host to host. For example, BAC can carry about 200 kb, YAC 0.2-2 Mb, and PAC 130-150 kb at maximum respectively. Even though spotting DNA directly onto the slide is the simplest and most accurate way, it is not always easy in practice. Firstly, since BAC is single copy, yields of BAC DNA are low. Secondly, high-molecular DNA sized more than 100 kb from BACs can be viscous, blocking the spotting needles intermittently. Therefore, researchers have to do large amount bacterial cultures and sonicate the DNA to reduce the molecular weight down (Pinkel *et al.*, 1998; Cai *et al.*, 2002). To overcome these limitations again, new technique was adopted; extract small amount of BAC DNA automatically from large number of small scaled culture and amplify them by using whole genome amplification such as, ligation mediated PCR (Snijders *et al.*, 2001; Klein *et al.*, 1999) or degenerated oligonucleotide primed (DOP) (Hodgson *et al.*, 2001; Veltman *et al.*, 2002). Another new approach enabling construction of arrays from minute amount of DNA is rolling circle amplification (Dean *et al.*, 2002). This rolling circle amplified products were proved to be a suitable resource for A-CGH (Smirnov *et al.*, 2004). This approach could also be used for small amount of tissue (Lage *et al.*, 2003; Paris *et al.*, 2003). These novel techniques are to amplify target sequences in BAC clones nonspecifically across the whole genome, therefore it could represent genomic complexity. DOP amplification is the most commonly used method for array preparation nowadays. Figure 2 represents the example of DOP primer and amplification of BAC DNA by DOP PCR. Recently, species specifically designed DOP PCR was developed enabling more specific amplification of target sequences and minimizing match with bacterial DNA at the same time, which can make array CGH result more valid (Fiegler *et al.*, 2003; Chung *et al.*, 2004). There is a new technique minimizing repetitive sequences with primers targeting for non-repetitive sequences only (Buckley *et al.*, 2002). Using this approach, repeat-free sequences are amplified by PCR from genomic clones and are spotted in pools as targets on a slide. DNA copy number changes can also be detected using arrays made from cDNAs or oligonucleotides (Lucito *et al.*, 2000). The cDNA arrays have proven their ability to detect large copy number changes like amplification, but the actual genomic resolution of the boundaries of single copy number change, especially focal single copy change is considerably less than that of BAC arrays.

A. Structure of DOP primer

6MW: CCGACTCGAGNNNNNNATGTGG (Telenius *et al.*, 1992)

B. Amplification of BAC DNA with DOP primer

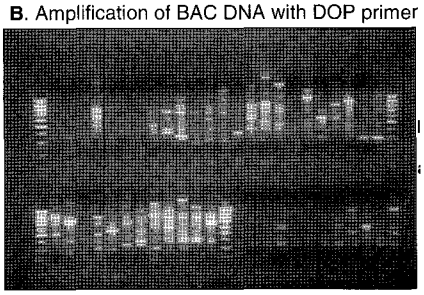

Fig. 2. Structure of a universal DOP primer (Telenius *et al.*, 1992) (a) and the examples of genomic amplification using DOP primer (b).

The resolution of BAC array has considerably improved since the first application of genome-wide array CGH for tumor analysis. Dumanski group constructed tile path array for chromosome 22 and analyzed some congenital and neoplastic diseases (Buckley *et al.*, 2002). In 2003, human BAC array with proper 1 Mb resolution has developed (Fiegler *et al.*, 2003) and recently full coverage tile path human BAC array was completed (Ishkanian *et al.*, 2004). In mouse study, Hodgson *et al.* applied mouse BAC array for pancreatic islet cell tumor analysis (2001). Cai *et al.* made BAC array with 3 Mb interval (2002) and Chung *et al.* improved the resolution to 1 Mb level (2004).

Recent development of bioinformatics tools for accurate identification of aneuploidy breakpoint and smoothing of A-CGH data is another optimistic sign of further standardization and application of A-CGH for medical researches (Jong *et al.*, 2004; Myers *et al.*, 2004; Wang *et al.*, 2004). Figure 3 demonstrates the example of identification of breakpoint using aCGH-Smooth software (Jong *et al.*, 2004). Because of these outstanding progress of A-CGH technology, more than 150 research papers using A-CGH have been published during past 6 months.

Applications of A-CGH to medical research

The easiest application of A-CGH is detection of multi-copy gain of DNA extracted from homogeneous cell lines. In this case, both test and reference cells are pure and the test DNA has much more genetic materials than reference. In many studies adopting A-CGH, genomic amplification is more commonly detected than single copy deletion (Hodgson *et al.*, 2001; Snijders *et al.*, 2001; Cai *et al.*, 2002; O'Hagan *et al.*, 2002). Figure 4

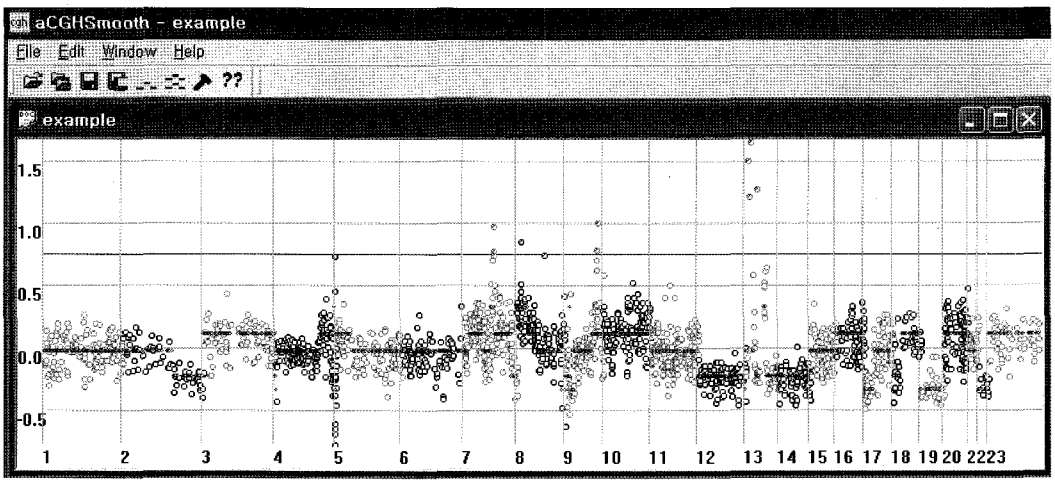

Fig. 3. Example of chromosome breakpoint identification using one of the bioinformatics tools for A-CGH analysis, aCGH-smooth software (http://www.few.vu.nl/~vumarray/).

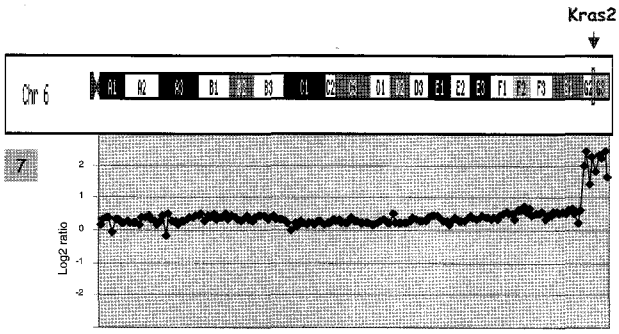

Fig. 4. Identification of *k-ras* oncogene amplification in murine mammary tumor. Red arrowhead indicates the amplification region containing *Ras*.

represents the precise detection of *Ras* amplification by A-CGH in murine mammary tumor. Detection of single copy number change is relatively hard, especially in narrow regions. In single copy deletion, in principle, signal intensity of test DNA is reduced 50% than reference DNA; and single copy gain makes 1.5 times more intense signal. The result could be obscured due to multiple factors discussed below. Since the major proportion of human genome is repetitive sequences, it is important to block repetitive sequences for reducing background noise and obtaining valid hybridization result in genomic DNA arrays comparing to expression array. As well as blocking repetitive sequences, multiple factors like normal cell contamination, non-specific interaction of labeled DNA to glass surface, uneven hybridization, washing condition, scanning variation, data normalization can affect the result significantly. Therefore, it is important to establish adequate controls and arrange every step carefully beforehand to detect valid single copy number change.

1. Application of A-CGH to cancer genomics

The enhanced resolution and reproducibility of A-CGH compared with chromosome CGH has been demonstrated by the fact that A-CGH could find the subtle copy number aberrations that were not detected by chromosome CGH. For example, in breast cancer, amplification found in 20q13.2 and *CYP24* gene was proved as oncogene for breast cancer after analysis (Albertson *et al.*, 2000). In neurofibromatosis type2 patient, frequency and boundary of genetic deletion in 22q was found accurately by A-CGH (Buckley *et al.*, 2002). Recently, genetic alterations in wide-spectrum of tumors have been analyzed using A-CGH. Numerous novel amplifications have been found in pancreatic cancer, osteosarcoma, fallopian tube carcinoma, and head and neck cancers (Redon *et al.*, 2002; Snijders *et al.*, 2003; Holzmann *et al.*, 2004; Man *et al.*, 2004). Whole chromosome profiling of lymphomas and gastrointestinal tumors were also comprehensively analyzed (De Leeuw *et al.*, 2004; Peng *et al.*, 2004). A-CGH can be used to analyze genome-widely for each stage of tumor genesis.

Animal disease models are actively studied using A-CGH. Deletions in chromosomes 6, 8, and 4 (12p11-p13, 16q24.3, 13q11-32 in human) and amplifications in chromosomes 2 and 4 (20q13.2, 1p32-36 in human) were found by A-CGH in mouse pancreatic islet cell tumor. Among genes in these regions, there are several candidates for tumor suppressor genes and oncogenes like *CYP24, PFDN4, STMN1, CDK1B, PPP2R3* and *FSTL1* (Hodgson *et al.*, 2001). The genomic instability affected by telomere dysfunction was studied in colon and breast cancers by using A-CGH (O'Hagan *et al.*,

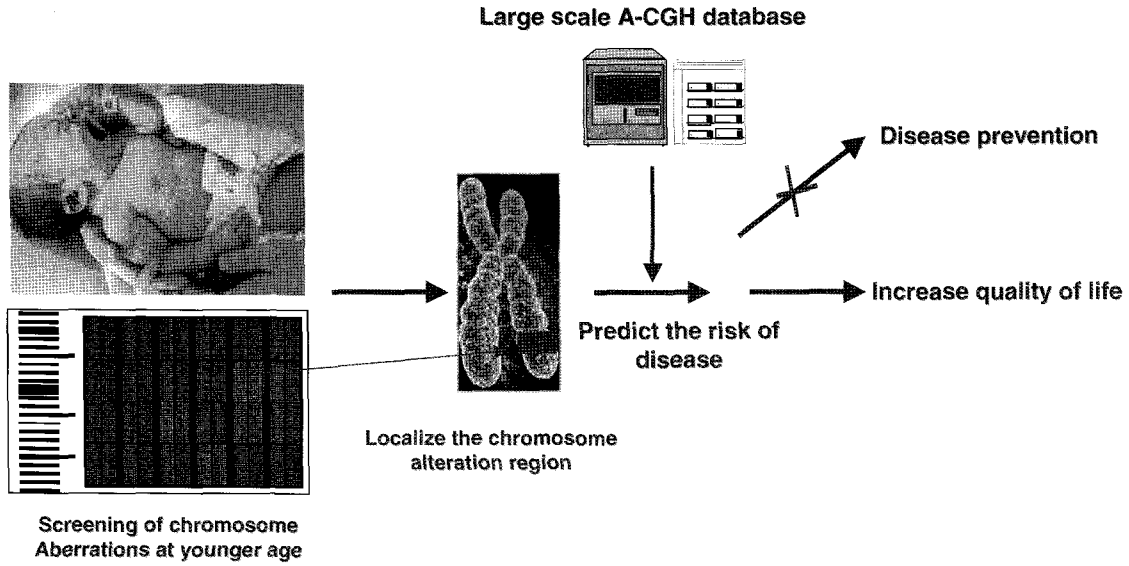

Fig. 5. Prospect of future biomedical application of A-CGH.

2002). As a result, minimal amplification region (MAR) was found and regional amplification or loss induced by nonreciprocal translocation due to telomere dysfunction proved to be one of the important tumorigenesis mechanisms. A-CGH analysis of murine neurolastoma revealed important ideas to understand human tumor progression (Hackett *et al.*, 2003).

2. Application of A-CGH to unknown congenital genetic disease

It is well-known that a variety of quantitative changes in genetic material underlie many congenital anomalies or mental retardations. In practice, several cytogenetic techniques have been used to diagnose these disorders. If the genetic changes are already identified, we can use karyotyping or FISH to confirm genetic abnormalities such as aneuploidy, regional loss or gain, translocation. But, there is a limitation to use existing molecular genetic techniques for detecting novel or subtle changes because of technical difficulties as we mentioned before. Even though causative genetic abnormalities for several congenital diseases like Down syndrome are already found, genetic changes causing most of congenital anomalies and mental retardation are still largely unknown. There are evidences that submicroscopic telomeric rearrangement is related with mental retardation. Also the role of subtelomeric rearrangement has been revealed directly and indirectly, but not fully proven. Recent researches using A-CGH for congenital diseases have been finding clinically important information. For example, new A-CGH designed to analyze telomeric or subtelomeric region is now being used for studying some idiopathic mental retardation (Veltman *et al.*, 2002; Knight *et al.*, 2000). A-CGH not only confirmed previous hypotheses for mental retardations of unknown origin, but also found novel changes (Veltman *et al.*, 2002). Indeed several interesting cryptic rearrangement, deletions, or duplications were detected in subtelomeric region of chromosome 1, 4, 9, 15 in idiopathic mental retardation (Harada *et al.*, 2004; Shaw-Smith *et al.*, 2004). Other types of complex neurobehavioral disorders or unknown spontaneous miscarriage were also studied using A-CGH, revealed some causative rearrangements or imbalances (Schaeffer *et al.*, 2004; Wang *et al*, 2004). Even though this field is relatively new and it needs more improvement, A-CGH based genetic diagnosis will become clinically important. To see more examples, A-CGH revealed the deletion boundary around 22q11.2, known as an important region for DiGeorge syndrome (Snijders *et al.*, 2001). A-CGH have uncovered important changes for cardio-facio-cutaneous syndrome and other congenital disorders like congenital aural atresia (Rauen *et al.*, 2002; Veltman *et al.*, 2002; Gunn *et al.*, 2003).

Prospects of A-CGH for biomedical research

CGH is expected to contribute to biomedical study enormously. Cancers are caused by multiple genetic changes sporadically as well as congenitally. It has been widely accepted that on or off of a single gene cannot explain complicated processes of tumor initiation,

development or metastasis. To understand the complex network of genetic alterations from single genes to genome, in addition to global gene expression profiling, one of the key information will be comprehensive genome-wide chromosome aberration data. To overcome the limitations of A-CGH such as tumor cell purity or isolation of single tumor clone, microdissection based A-CGH analysis can be more useful. This microdissection and whole genome amplification based approach is already applied as an alternative way to increase the tumor cell purity and to use extremely small number of cells for A-CGH. Using A-CGH we can understand more about idiopathic genetic diseases, psychiatric diseases, and metabolic disorders. There have been evidences of chromosomal alterations in type 1 diabetes mellitus and autoimmune disease like SLE. Ultimately, as shown in figure 5, we hope to use the accumulated A-CGH database to predict these diseases in younger age and prevent them.

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